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A RECEPTOR-COUPLED EVANESCENT BIOSENSOR

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An acetylcholine receptor-based optical biosensor was developed that uses the evanescent wave to excite fluorescein isothiocyanate-labelled α-bungarotoxin (FITC-α-BGT). Fluorescence was trapped by and propagated back up the fiber. Pure nicotinic acetylcholine receptor (nAChR) protein, isolated from Torpedo electric organ, was immobilized noncovalently on quartz optic fibers. Specific FITC-α-BGT binding to the nAChR protein on the optic fibers was inhibited by agonists and antagonists of the nAChR and was insensitive to high salt concentrations. Its specificity, its sensitivity, the absence of drift problems, its user friendly nature, the known simple mechanism underlying signal production, the very high signal-to-noise ratio, and its ability to detect specific receptor ligands in a solution of high ionic strength are clear advantages of this sensor.

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PREFACE

The work described in this report was authorized under Contract No. DAAA15-89-C-0007. This work was started in March 1989 and completed in October 1989. The experimental data are recorded in laboratory notebooks in the possession of the contractor.

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A RECEPTOR-COUPLED EVANESCENT BIOSENSOR

1. INTRODUCTION

Neurotransmitter and hormone receptors serve as biosensors of specific chemical signals that range from amino acids (e.g., γ-aminobutyric and glutamic acids) to peptides (e.g., insulin). With the exception of steroid hormone receptors, these chemoreceptors are usually located in the cell membrane where they monitor the external environment and respond to the presence of specific chemicals. The receptor proteins normally have high affinities for their specific hormones or neurotransmitters. The binding of these chemicals is followed by a transmembrane-signalling mechanism that triggers a cellular response. Receptor-based biosensors are detection devices that utilize a receptor protein as their biological sensing element, and this is intimately connected to a physical-chemical transducer.

Evanescent fluorosensors, with an appropriate indicator, can provide a specific and sensitive transducer for a receptor-based biosensor. Although several reports have appeared in the literature using antibody-antigen based evanescent fluorosensors,[1,2] this is the first report where a neurotransmitter receptor protein is coupled to an optic fiber and is used to detect receptor ligands. If successfully used with various receptors, these optic sensors may become very useful analytical devices for drugs and toxicants that bind to receptors and may develop into significant diagnostic devices. In the case of the fiber-optic system reported herein, the cylindrical waveguide evanescent fluorosensor makes use of the evanescent wave effect by exciting a fluorophore just outside the waveguide boundary.[3-5] A portion of the resultant fluorophore emission then becomes trapped in the waveguide and is transmitted back up the fiber. This sensing technique is particularly well suited to a receptor-based biosensor. This is because a fluorescent-tagged ligand bound to a receptor protein, which is immobilized at the fiber surface, can be monitored without interference from the probe in the bulk solution.

Receptor proteins are identified in subcellular preparations either by their specific high affinity binding of specific probes or by their expressed function. For example, it is common to use specific radiolabelled α-bungarotoxin (α-BGT) binding as an indicator for a skeletal muscle type nicotinic acetylcholine receptor (nAChR), just as it is common to think of propranololsensitive isoproterenol-induced formation of c-AMP as indicative of the presence of β-adrenergic receptors.

The nAChR was the first neurotransmitter receptor to be purified and its molecular properties elucidated.[6-9] The receptor genes have been cloned[10,11] and it is likely to be the first receptor protein that may be produced in large quantities for commercial application. Because the nAChR can easily be harvested in milligram quantities from electric organs of electric fish,[12] a few laboratories are already experimenting with nAChR-based biosensors.[13-15] In an earlier study, we noncovalently immobilized this nAChR (in asolectin lipids) onto the surface of a planar interdigitated capacitive sensor and were

able to detect specific concentration-dependent increases in capacitance when an agonist was present.[14] This increase was inhibited by antagonists. Although the biosensor could be regenerated, there was great variability in the response depending on the engineering of the capacitance device (unpublished data). Also, several events may have been responsible for the increased capacitance, including binding of agonist to the receptor, which produces changes in receptor conformation and opening of the receptor's ionic channel resulting in cation flux through it. The present study was initiated to develop and evaluate another detection device. A fiber optic evanescent fluorosensor and fluorescein-labelled α-BGT were utilized to provide evidence that the nAChR adsorbed onto the surface of quartz optic fiber could detect ligands that bind to this receptor, whether agonist (e.g., nicotine) or antagonists (e.g., α-BGT or the skeletal muscle relaxant pancuronium). The mechanisms of transduction were simple and the change in signal transduction depended only on the receptor-ligand binding step.

2. MATERIALS AND METHODS

2.1 Purification of Nicotinic Receptor.

The nAChR was purified as previously described.[8] Torpedo electric organ (400 g) was homogenized in one liter of buffer (5 mM Tris, 154 mM NaCl, 1 mM EDTA and 0.1 mM phenylmethylsufonyl fluoride, pH 7.4) in a stainless steel Waring blender. The homogenate was filtered through 4 layers of cheesecloth and the filtrate centrifuged at 30,000 x g for 60 min. The pellets were suspended in 90 mL of the same buffer containing 1% Triton X-100 and shaken at 4°C for 30 min, so as to solubilize the receptor protein. The suspension was then centrifuged at 105,000 x g for 60 min and the supernatant fraction collected. This Triton X-100 extract was mixed with 40 g of the cobra Naja α-neurotoxin affinity Sephadex gel and incubated for 2 h at 23°C. The incubation mixture was filtered and the affinity gel washed with the homogenization buffer containing 0.1% Triton X-100. The affinity gel was then mixed with 50 mL of 1 M carbamycholine for 4 h at 23°C, then filtered, and the filtrate, containing carbamylcholine and the nAChR protein, was dialyzed against 5 mM Tris pH 7.2 to remove the drug and most of the detergent.

2.2 Preparation of Fluorescein Labelled α-Bungarotoxin.

α-BGT was obtained from Ventoxin Laboratories, Frederick, MD. Coupling of α-BGT to fluorescein isothiocyanate (FITC) was performed as described by Suszkiw & Ichiki[16] with minor modifications. α-BGT (2 mg) was reacted with 1 mg FITC on celite (Sigma) in 1 mL of 50 mM bicarbonate buffer at pH 9.5 for 15 min. The celite was removed by centrifugation and the supernatant loaded onto a Sephadex G-25 column (25 x 1.1 cm) and developed with 5 mM ammonium acetate, pH 5.8. The void fractions were pooled, lyophilized, resuspended in 50 mM ammonium acetate, pH 5.8, and loaded onto a CM-52 column (10 x 1.5 cm). The column was developed with the same buffer until the first peak of fluorescence had cleared the column, after which the remaining fluorescent material was eluted with 0.5 M ammonium acetate. Fractions from the second peak were pooled, lyophilized, dissolved in water, and assayed for binding to the nAChR. This was detected by

its ability to inhibit carbamylcholine-stimulated binding of the allosteric inhibitor [^{3}H]perhydrohistrionicotoxin, as previously reported.[17] The fluorescein isothiocyanate labelled α-bungarotoxin (FITC-α-BGT) had an excitation maximum at 493 nm and an emission maximum at 516 nm. FITC-α-BGT at 0.5 μM in phosphate buffered saline (PBS) (154 mM NaCl in 10 mM sodium phosphate buffer pH 7.4) yielded relative fluorescence (measured on a Gilford Fluoro IV) that was 5 times that of an equimolar solution of FITC in PBS.

2.3 Immobilization of Receptor Protein.

Pure nAChR was noncovalently immobilized on the quartz fibers during a 12-hour incubation in a solution which contained 50 μg/ml nAChR protein in 10 mM sodium phosphate buffer, pH 4.0. Excess receptor was removed from the fiber by one passage through 5 mL of 1 mM sodium phosphate, pH 7.4 or PBS, as specified in Results.

2.4 Apparatus.

All experimental work was carried out using a fluorimeter designed and built at ORD, Inc., North Salem, NH. The fiber optic evanescent fluorosensor apparatus (Figure 1) was similar in configuration to that reported by Glass, et al.[5] Components of this instrument included a 10W Welch Allyn quartz halogen lamp, a Hamamatsu S-1087 silicon detector, an Ismatec fixed speed peristaltic pump, a Pharmacia strip chart recorder, and bandpass filters and lenses as indicated in the schematic. The quartz fibers, 1 mm in diameter with polished ends, were obtained from ORD, Inc.

The fiber optic evanescent fluorosensor made use of the evanescent wave effect by exciting a fluorophore just outside the waveguide boundary (excitation wavelength = 485/20 nm). A portion of the resultant fluorophore emission then became trapped in the waveguide and was transmitted back up the fiber. This was detected after transmission through 510 nm LP and 530/30 nm filters. The flow cell allowed the center 47 mm of a 60 mm long fiber to be immersed in 46 μl which was exchanged every 14 sec.

2.5 Fluorescence Measurements.

After immobilization of the nACh receptor on the quartz fiber, the fiber was placed in the instrument and perfused for 5 min with PBS containing either bovine serum albumin (BSA) (0.1 mg/mL) alone or BSA containing a receptor ligand where indicated. The fiber was then co-treated with FITC-α-BGT (5 nM) along with the appropriate ligand in PBS containing BSA (0.1 mg/mL). Between experiments, the flow cell was washed with 1% SDS for 2 min followed by PBS for 10 min. Initial rates were determined graphically from tracing of the mV response vs. time.

2.6 ^{125}I-α-BGT Binding.

The specific activity of ^{125}I-α-BGT (100 Ci/mmol, from New England Nuclear) was adjusted with unlabelled α-BGT. Quartz fibers with and without immobilized nAChR were pretreated for 5 min with PBS containing 0.1 mg/mL BSA.

For determination of total α-BGT binding sites, fibers were incubated in the presence of 300 nM ^{125}I-α-BGT for 1 h, washed by 3 passages in PBS, and the radioactivity counted in a LKB Autogamma counter. Inhibition of initial rates of ^{125}I-α-BGT binding by d-tubocurarine (d-TC) were measured using pretreated (5 min in 0.1 mg/mL BSA in PBS) nAChR-coated fibers. These fibers were incubated for 2 min in 5 nM ^{125}I-α-BGT which bound to quartz fibers lacking immobilized receptors, and was typically 5-10% of the total binding.

3. RESULTS

The initial rates of binding of FITC-α-BGT to untreated quartz fibers showed a dose-dependent relationship (Figure 2, A). These rates were similar for either untreated quartz fibers or quartz fibers coated with immobilized nAChR protein. However, FITC-α-BGT binding to untreated quartz fibers was either partially inhibited (at 500 nM FITC-α-BGT) or completely inhibited (at 5 nM FITC-α-BGT) by pretreatment of the fibers with BSA (0.1 mg/mL) (Figure 2; Table 1). Furthermore, pretreatment of the nAChR-coated fibers for 30 min with 5 nM unlabelled α-BGT resulted in 75% inhibition of the initial rate of FITC-α-BGT binding (Table 1). These results suggested that when nonspecific FITC-α-BGT binding was eliminated by treatment of the fibers with BSA, the specific binding of FITC-α-BGT to the nAChRs (noncovalently immobilized on these fibers) was responsible for the observed fluorescence. The fluorescence response of the receptor-coated fibers increased for 30 min then remained stable for several hours.

The nAChR-coated optic fibers bound ^{125}I-α-BGT in a dose-dependent manner (Figure 3A). Increasing ratios of ^{125}I-α-BGT to unlabelled α-BGT resulted in a linear increase in the amount of ^{125}I-α-BGT bound/fiber. The receptor-coated fibers bound 4.2 $\pm$ 0.4 pmol ^{125}I-α-BGT sites/fiber (n = 9). Increasing the ratios of FITC-α-BGT to nonlabelled α-BGT, at a total concentration of 3 μM, also yielded a linear increase in the maximum fluorescence response (Figure 3, B). The fluorescence response for the nAChR-coated fibers saturated after 30 min and yielded a maximum value of 8.4 V/fiber for FITC-α-BGT. The noise level of the fluorescence response during a typical experiment was in the range of 10-50 mV. This translates into a maximum sensitivity of 25 fmol of nAChR which could be detected on a fiber, and a signal to noise ratio of >99.

The initial rate of FITC-α-BGT (5 nM) binding to the receptor-coated fibers was inhibited in a dose-dependent manner by the receptor's standard competitive antagonist d-tubocurarine (d-TC) (Figures 4 and 5). However, about 20% of the maximum binding remained even at 1 mM d-TC. The initial rate of ^{125}I-α-BGT (5 nM) binding to the nAChR-coated fibers was also inhibited by d-TC (Figure 5). The displacement curves were similar, though that for FITC-α-BGT was shifted to the right. This was presumably due to the effect of the residual rate of FITC-α-BGT binding even at high concentrations of d-TC.

α-BGT, d-TC, and the agonist carbamylcholine, all of which bind to the same receptor site, inhibited binding of FITC-α-BGT to the nAChR-coated fibers, and resulted in a dose-dependent decrease in fluorescence (Figure 6). The relative potencies of d-TC, carbamylcholine and unlabelled α-BGT in inhibiting the

initial rates of binding of FITC-α-BGT (5 nM) to the fiber-coated nAChR, were similar to those reported for detergent extracts of the purified nAChR from Torpedo.[18,19] However, the IC_{50} (concentrations giving 50% inhibition) values for d-TC and carbamylcholine were shifted an order of magnitude to the right of that expected for detergent extracts of purified nAChR. Furthermore, when the receptor-coated fibers were pretreated with 1 mM carbamylcholine for 10 min prior to measuring FITC-α-BGT displacement, it gave 42% inhibition (Table 3) instead of only 10% (Figure 6) when the fibers were not pretreated.

The initial rates of FITC-α-BGT binding to nAChR were not affected by concentrations of NaCl up to 154 mM (Table 2). Similarly, inhibition of FITC-α-BGT binding by d-TC was also unaffected by ionic strength. On the other hand, the initial rates of FITC-α-BGT binding to nAChR-coated fibers were sensitive to cholinergic ligands (Table 3). Several receptor agonists and antagonists effectively blocked the binding of FITC-α-BGT and reduced the rate of increase in observed fluorescence.

4. DISCUSSION

The intensity of the observed fluorescence is dependent on the number of fluorescent molecules within the boundaries of the evanescent wave (i.e., about 1000 Å from the fiber surface). Fluorescein isothiocyanate at 5 nM in PBS is not detected by the fluorosensor apparently because fluorescein does not bind that well to quartz glass. However, 1 nM FITC-α-BGT, a concentration which gives equivalent fluoroescence to 5 nM FITC solutions (measured on a Gilson Fluoro IV spectrofluorometer), produces a good fluorescence signal from the evanescent fluorosensor. Using the slope of the initial change in fluorescence as a parameter, it is evident that FITC-α-BGT binds to the quartz fiber in a dose-dependent manner (Figure 2). In the presence of 0.1 mg/mL BSA, this non-specific binding of 5 nM FITC-α-BGT to the quartz fiber is totally eliminated (Figure 2, A and B). This is a concentration of BSA that does not interfere with binding of FITC-α-BGT to the nAChR (Figure 2, B; Table 1). Accordingly, it may be concluded that the observed fluorescence in the BSA-treated fibers is a result of FITC-α-BGT binding to the nAChR and not the result of its nonspecific binding to the quartz fiber.

The binding of α-BGT to the nAChR is a quasi-irreversible event, making it difficult to study competition displacement of α-BGT binding by reversibly binding drugs under equilibrium conditions. Therefore, affinities of receptor agonists and antagonists have been measured accurately by monitoring their effects on the association rate of α-BGT at pre-equilibrium conditions.[18,19] These reversible ligands, at physiological concentrations, have been shown to decrease the rate of toxin binding in a dose-dependent manner.

This optical biosensor may also be used to quantitate nAChR protein in solutions. The amount of nAChR picked by the optic fiber, determined from ^{125}I-α-BGT binding experiments (Figure 3, A), is either 4.2 pmol/fiber or 29 fmol/mm^2. This value is similar to that reported by Bhatia, et al.,[20] for covalent immobilization of IgG to silica fibers (i.e., 6 fmol/mm^2). The maximum fluorescence given when all receptor sites bind FITC-α-BGT is 8.4 V/fiber

Figure 3, B). Considering the very small noise level of less than 1% of the maximum signal (i.e., 10-50 mV), as little as 25 fmol of receptor per fiber are sufficient to give a significant signal. This means that as little as 6.2 ng of receptor protein per fiber can be detected.

The finding that binding of FITC-α-BGT to the immobilized nAChR on the optic fiber is affected by nicotinic cholinergic ligands (Table 3; Figures 4-6) indicates that the recognition function of the nAChR protein is intact. Consequently, this technique is applicable for detection of any cholinergic ligand which competes for the α-BGT binding site. The values of IC_{50} for d-TC and carbamylcholine are about an order of magnitude higher on binding of FITC-α-BGT compared to their IC_{50} values on binding of ^{125}I-α-BGT to detergent extracts of Torpedo nAChRs[21] (i.e., d-TC 6.4 μM, carbamylcholine 50 μM). These results suggest a change in the fiber-immobilized receptor, which may be due to the fact that the nAChR fluorosensor utilizes pure receptor protein in absence of detergents or lipids of its normal membrane environment. It may be possible to enhance the sensitivity of the sensor to cholinergic ligands by severalfold if the environment of the nAChR on the fiber is lightly modified, e.g., by addition of lipids after receptor immobilization on the optic fiber. The observed higher potency of 1 mM carbamylcholine in inhibiting the signal (42%) when the fiber is preexposed to carbamylcholine (Table 3 vs. Figure 6) may be a result of receptor desensitization.[18]

The optic fiber nAChR biosensor shows high sensitivity towards α-BGT, detecting concentrations less than 1 nM, compared to >50 nM for another biosensor using an electrode transducer.[15] Another important advantage is that the mechanism underlying signal production in this receptor-based optical biosensor is clearly understood, while the mechanism is unknown for three others; the ion-sensitive field-effect transistor,[13] the capacitive sensor[14] and the interdigitated electrode.[15] Furthermore, the optic-fiber biosensor has high signal-to-noise ratio of 99 and is stable in high ionic-strength media (1-154 mM) (Table 2). By contrast, the ion sensitive field effect transistor[13] and the interdigitated electrode[15] nAChR biosensors have a signal-to-noise ratio of only 0.1-0.2 and could operate only in very low ionic-strength media. The insensitivity of the fluorescent signal to high salt concentration is an important feature that would allow the biosensor to operate well in high ionic-strength media such as is found either in body fluids (e.g., plasma and urine) or the environment. Another advantage of the nAChR optic-fiber fluorosensor is that binding of α-BGT is a more receptor-specific signal than a response to acetylcholine in biosensor assays, especially when the mechanism of transduction is still unknown. Muscarinic receptor and acetylcholinesterase are two proteins that would react with acetylcholine and interfere with signal production in either capacitive or electrode sensors. In an earlier study, this factor was addressed by comparing pure and partially purified nAChR preparations.[14]

A disadvantage of using an irreversible FITC-labelled ligand is that the biosensor cannot be regenerated and reused because of the use of the quasi-irreversible α-BGT as the probe. Although a disposable sensor may be useful, another easily reversible fluorescent probe would allow multiple uses of the single fiber. Use of such probes is currently under investigation.

The utilization of a receptor-based biosensor to detect drugs and toxicants offers several advantages. First of all, it neither suffers from interference problems, as do existing immunosensors, nor does it have drift problems inherent in chemical and ion-sensitive, field-effect transistors.[22] Response time is fairly fast, with the assay completed in minutes. The assay is inexpensive and does not use hazardous material (e.g., radioactive ligands). It is also user friendly because it is rather easy to learn and apply.

Figure 1

Schematic presentation of the optical system used to measure fluorescence.

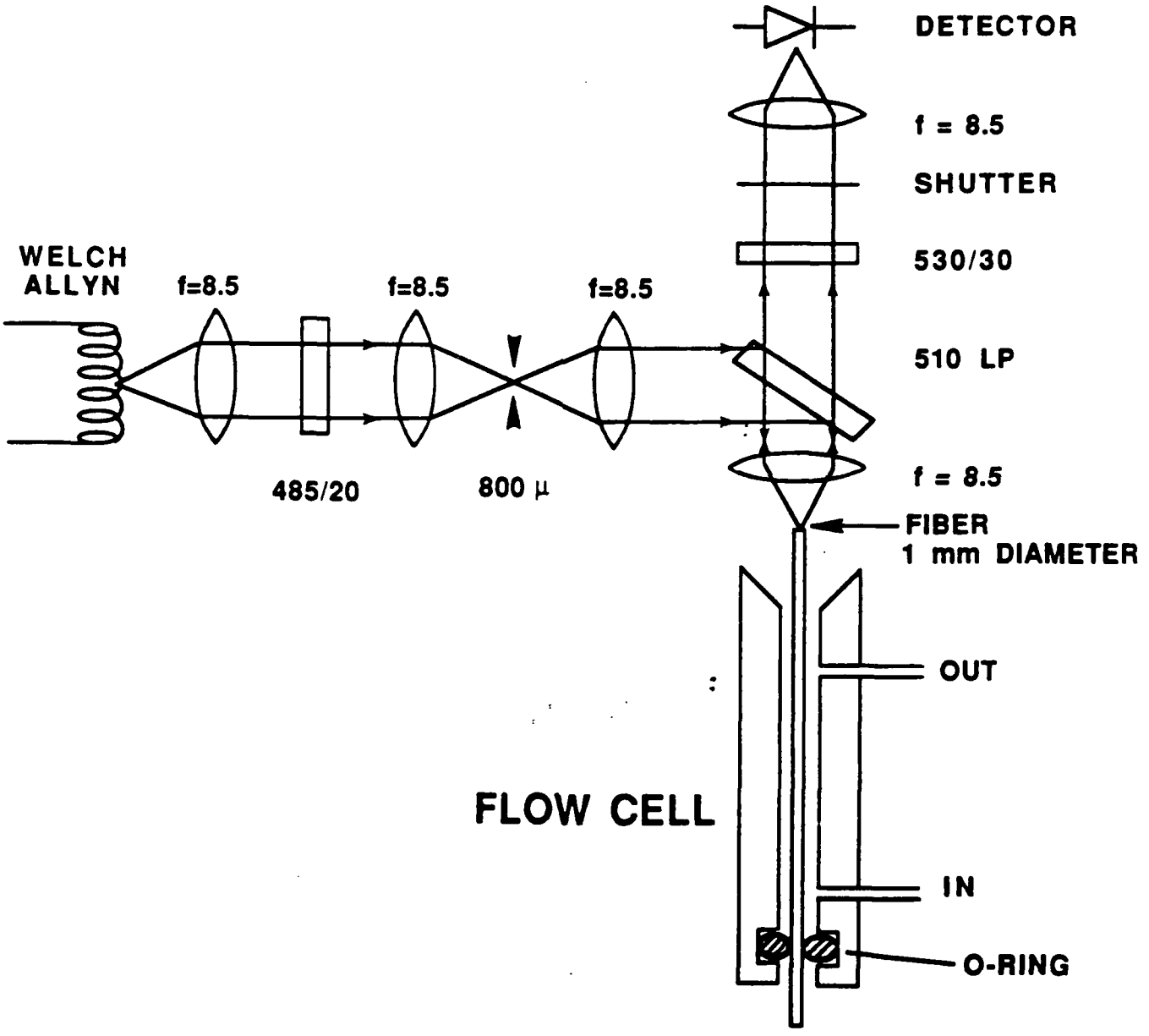
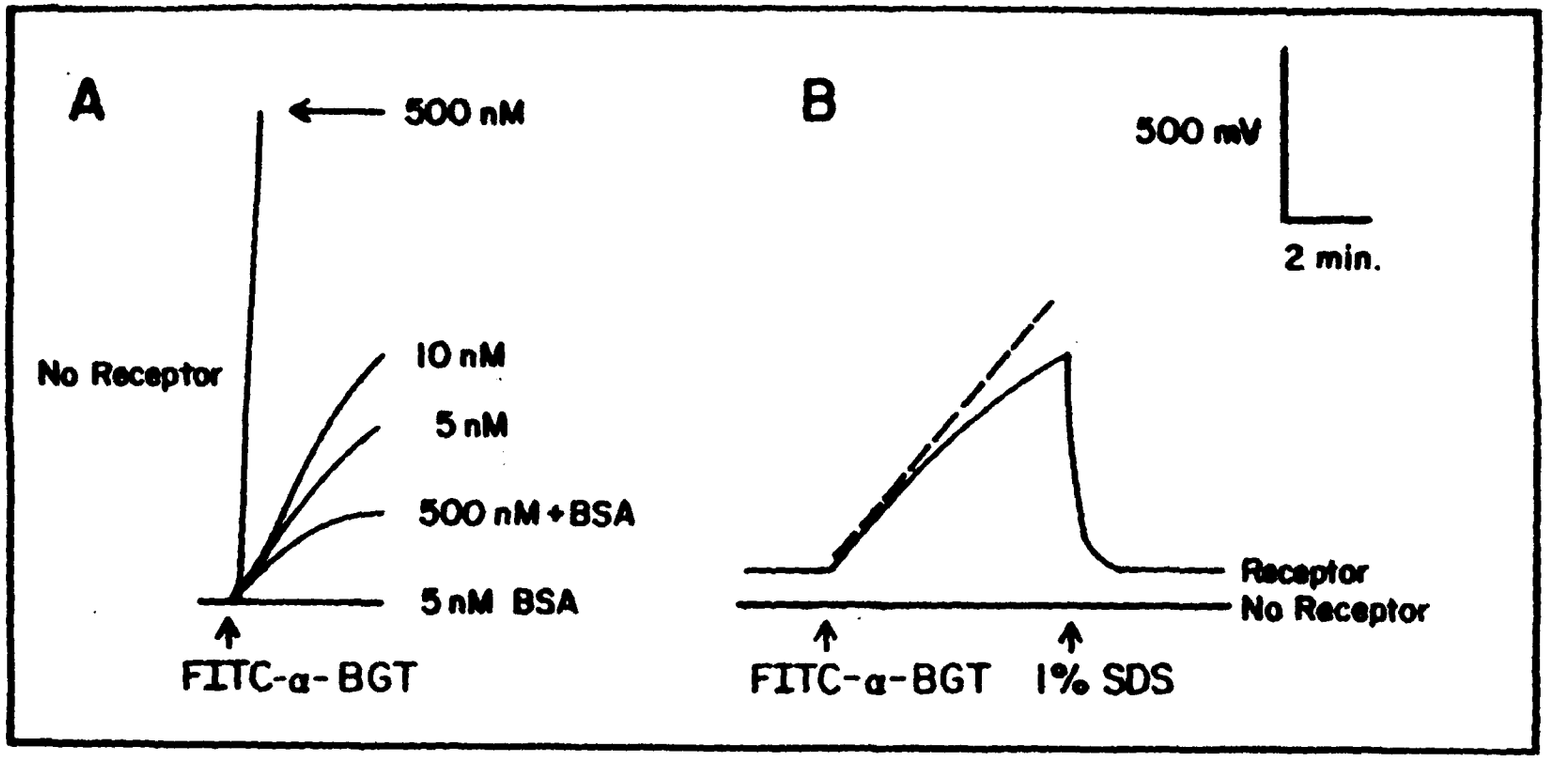


Figure 2

Binding of fluorescently labelled α-BGT to: A) untreated quartz fibers in the presence or absence of BSA (0.1 mg/mL in PBS buffer) and B) untreated or nAChR-coated quartz fibers.



FITC-α-BGT was introduced at 5 nM in PBS containing BSA (0.1 mg/mL. Dashed line represents graphically determined initial rates. Receptors and FITC-α-BGT were washed from the fiber with 1% sodium dodecyl sulfate (SDS).

Figure 3

Specific binding of α-BGT to nAChR-coated optic fiber. A) Binding of ^{125}I- α-BGT to fibers as a function of the specific activity of ^{125}I- α-BGT. Total α-BGT concentration was 300 nM. Symbols and bars represent means of triplicate determinations ± SEM. B) Maximum fluorescence response as a function of the ratio of FITC-α-BGT to nonlabelled α-BGT. Total α-BGT concentration was 3 μM. Data are representative of at least two measurements.

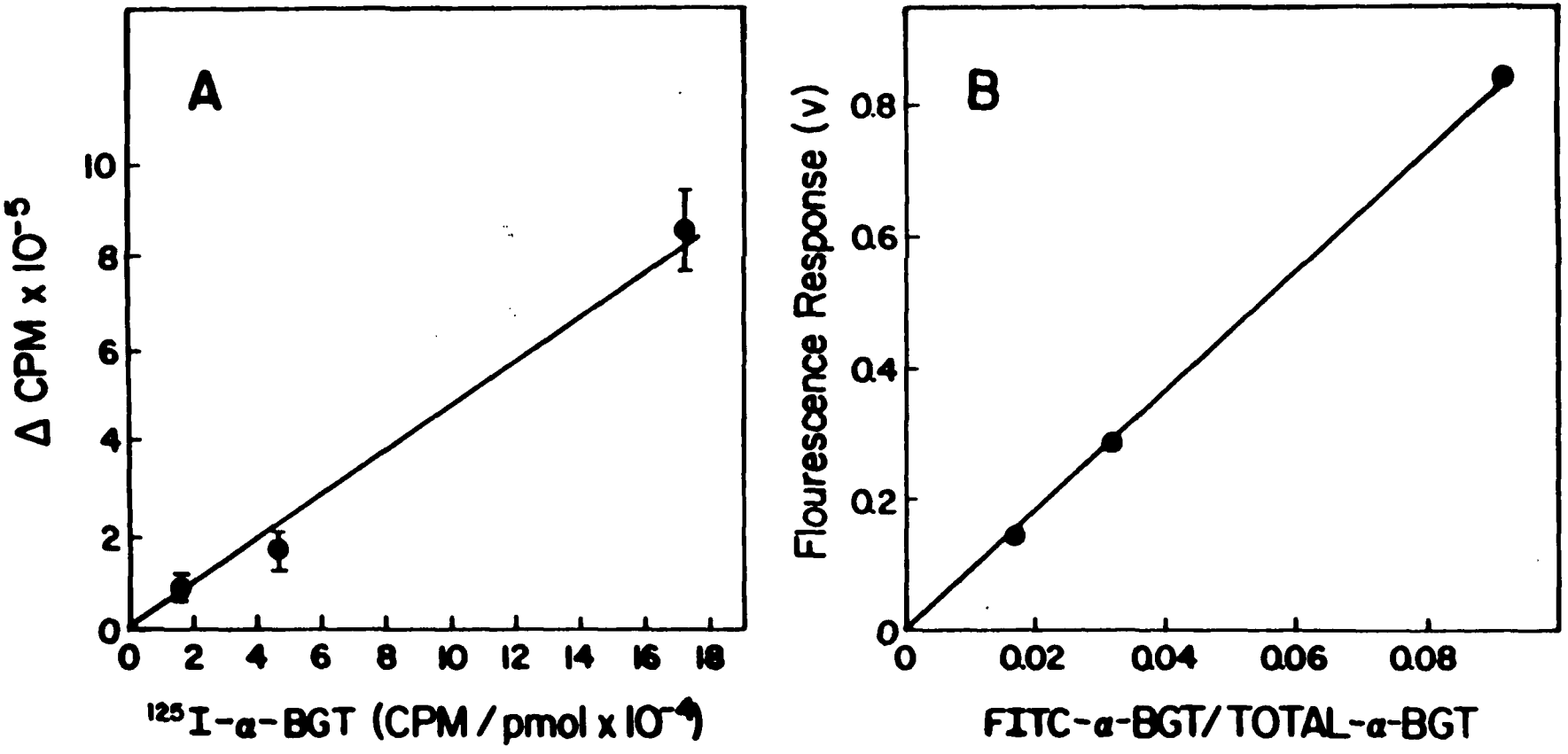


Figure 4

The concentration-dependent inhibition of receptor-mediated binding of FITC-α-BGT (5 nM) by the competitive antagonist d-TC.

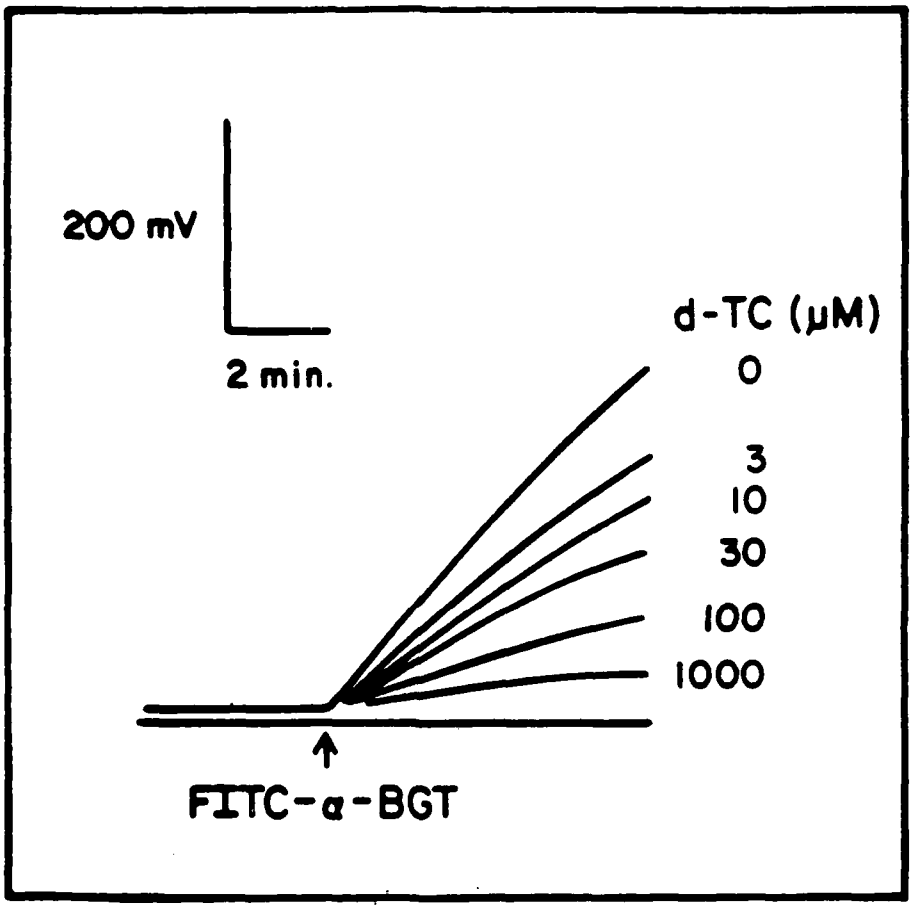


Figure 5

The effects of various concentrations of d-TC on the initial rates of binding of 5 nM of ^{125}I-α-BGT (●), and FITC-α-BGT (○) to nAChR-coated fibers. In both cases, the fibers were co-incubated with either d-TC or the labelled α-BGT. Symbols and bars are means of triplicate experiments ± SEM.

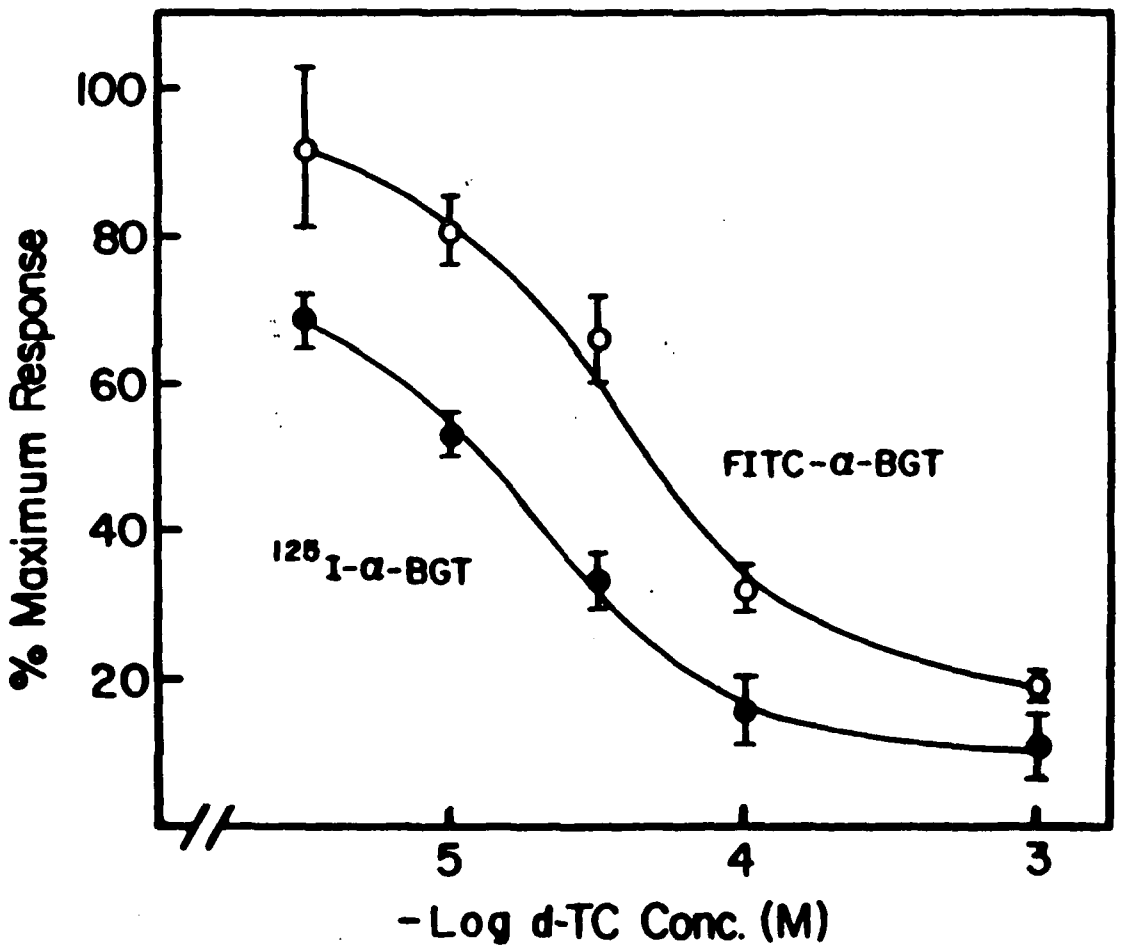
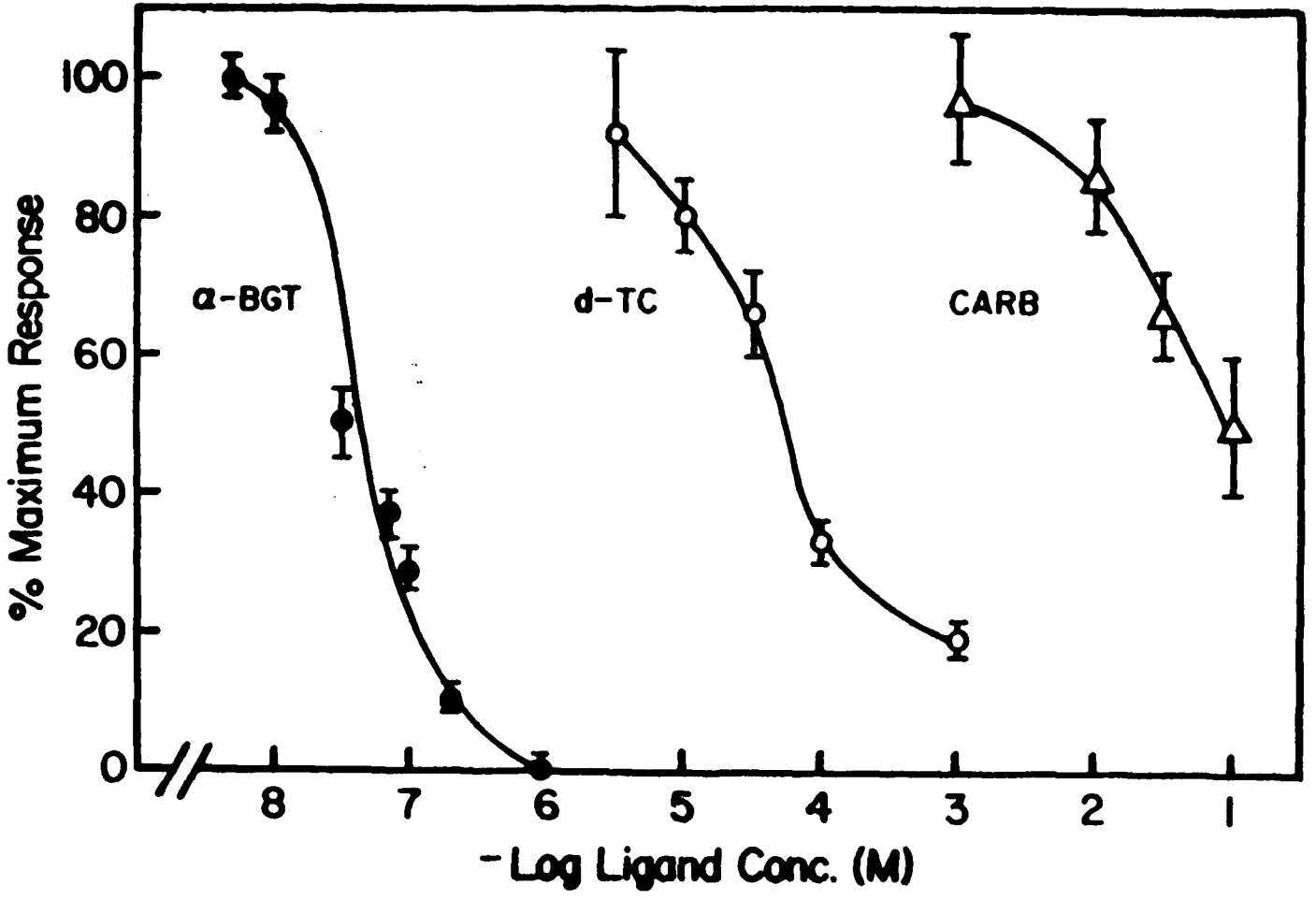


Figure 6

The effects of various concentrations of d-TC (○), carbamylcholine (△), and α-BGT (●) on binding of 5 nM FITC-α-BGT to nAChR immobilized on quartz fibers.



The various ligands at the appropriate concentrations were co-perfused with FITC-α-BGT. There was no premeasurement treatment of the fibers with ligands/BSA alone. Symbols and bars are means of triplicate measurement ± SEM.

Table 1. Effect of BSA pretreatment on nonspecific binding of FITC-α-BGT.

Fiber Type[a]	Treatment[b]	Initial Rate[c]
Quartz untreated	FITC-α-BGT	108
Quartz untreated	FITC-α-BGT, BSA	0
Quartz with nAChR	FITC-α-BGT	83
Quartz with nAChR	FITC-α-BGT, BSA	107
Quartz with nAChR	FITC-α-BGT, BSA (pretreated with α-BGT)	25

[a] Untreated fibers were washed with methanol prior to use. Quartz fibers were coated with nAChR, as described in "Methods."

[b] Fibers were perfused at 0.2 mL/min with PBS buffer containing FITC-α-BGT (5 nM), BSA 0.1 mg/mL, or pretreated with unlabelled α-BGT (5 nM, 30 min), as specified. Fibers treated with BSA were pretreated for 5 min prior to introduction of FITC-α-BGT.

[c] Initial rates were determined graphically from the first 30 s of data. Rates are representative of at least three experiments.

Table 2. Effect of ionic strength on FITC-α-BGT binding.

Na Phosphate Buffer[a] (mM)	NaCl (mM)	d-TC (μM)	Initial Rate[b] (mV/min)
10	154	0	103 ± 9
10	154	30	60 ± 10
1	0	0	113 ± 7
1	0	30	50 ± 5

[a] Buffer contained 0.1 mg/mL BSA. Fibers were pretreated for 5 min with the buffer indicated before introduction of 5 nM FITC-α-BGT in the appropriate buffer in absence or presence of d-TC.

[b] Rates ± SEM (n=3).

Table 3. Inhibition by receptor ligands of the binding of FITC-α-BGT to the nAChR on the optic fibers.

Drug[a]	% inhibition of initial rate of fluorescence change[b]
Agonists	
Acetylcholine	61
Carbamylcholine	42
Nicotine	27
Depolarizing blockers	
Decamethonium	52
Succinylcholine	45
Competitive antagonists	
d-Tubocurarine	87
Pancuronium	76
Naja α-neurotoxin (10 μM)	100

[a] Unless otherwise specified, the drug was present at 1 mM in PBS containing BSA (0.1 mg/mL) and FITC-α-BGT (5 nM). Fibers were pretreated with the indicated drug for 10 min prior to addition of the FITC-α-BGT.

[b] Initial rate of fluorescence change in absence of inhibitor = 100%. The values are means of two experiments which varied by < 10%.

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